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Assessment of the Antidiabetic Potential of an Aqueous Extract of Honeybush (*Cyclopia intermedia*) in Streptozotocin and Obese Insulin Resistant Wistar Rats

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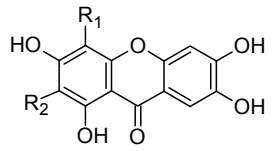
1. Introduction

It has been estimated that diabetes will affect 439 million adults by 2030, with the major increase occurring in developing countries (Shaw et al., 2010). It is projected that it will rank as the 9th leading cause of death in low-income countries (Mathers & Loncar, 2006). There are two major types of diabetes, i.e. type 1 (T1D) or insulin dependent diabetes and type 2 (T2D) or non-insulin dependent diabetes. The incidence of T2D is reaching epidemic proportions and has been associated with an increase in obesity (Venables & Jeukendrup, 2009). According to the World Health Organisation (WHO, 2011) the main complications associated with diabetes are cardiovascular disease and renal failure. Although genetic factors may play a role, life-style factors, such as reduced exercise and poor diet, specifically a high carbohydrate, high fat diet devoid or low in fruit and vegetables, have been shown to increase the risk of diabetes (Astrup, 2001).

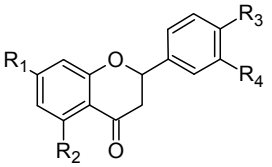
Medicinal plants have been used in folk medicine and traditional healing systems such as Ayurveda and Traditional Chinese Medicine (TCM) for the treatment of diabetes (T.S.C. Li, 2003; Modak et al., 2007; Singh et al., 2009; Yen et al., 2003). On the African continent as many as 90% of the populations of some countries relies on plants as the principal source of medicine for the treatment of different diseases, including diabetes (Hostettman et al., 2000), as they provide an affordable alternative to drugs. In South Africa a large number of plants, belonging to plant families such as the Asteraceae and Lamiaceae, amongst others, have been traditionally used for the treatment of diabetes (Deutschländer et al., 2009; Erasto et al., 2005; Thring & Weitz, 2006).

Globally, there is a movement towards alternatives to single chemical entities as favoured by the pharmaceutical industry. These alternatives are rationally selected, carefully standardised, synergistic traditional herbal formulations and botanical drug products which are supported by robust scientific evidence (Patwardhan & Mashelkar, 2009). In many instances the value of herbal and medicinal plant extracts lies not in a single compound, but in their complex phytochemical nature. These complex mixtures of often unspecified compounds are able to modulate multiple targets (Y. Li et al., 2008). Antidiabetic phenolic compounds in the extracts also have the ability to ameliorate oxidative stress (Han et al., 2007), an underlying mechanism to the pathogenesis of diabetes (Ceriello & Motz, 2004). One such compound is the xanthone *C*-glucoside, mangiferin (1,3,6,7-tetrahydroxy-xanthone-*C*2-β-D-glucoside), demonstrating antihyperlipidaemic, antihyperglycaemic and antioxidant properties (Wauthoz et al., 2007). Mangiferin (Fig. 1) was shown to protect against streptozotocin (STZ)-induced oxidative damage to cardiac and renal tissues in Wistar rats (Muruganandan et al., 2002). Its presence in the endemic South African *Cyclopia* spp. (family Fabaceae; tribe Podalyrieae) suggested the potential use of these plant species as antidiabetic nutraceuticals or even phytopharmaceuticals. Hesperidin (Fig. 1), another antioxidant and compound demonstrating hypoglycaemic properties in rodents (Akiyama et al., 2010; Jung et al., 2004), is also one of the major monomeric polyphenols present in *Cyclopia* spp. (Joubert et al., 2003). A decoction of *Cyclopia* spp. was used in the past as a restorative and as an expectorant in chronic catarrh and pulmonary tuberculosis. However, it is as the herbal tea, honeybush, that *Cyclopia* spp. are increasingly appreciated by consumers world-wide. The tea is primarily exported to the Netherlands, Germany, United Kingdom and United States of America. It is even exported to traditional tea-drinking countries such as Sri Lanka, India, Japan and China. Commercial herbal tea production comprised three main species, viz. *C. genistoides*, *C. intermedia* and *C. subternata*. Of these, *C. intermedia*, harvested almost exclusively from the wild, provides the bulk of honeybush production.



2, mangiferin
(R_1 = H; R_2 = *C*-β-D-glucoside)

3, isomangiferin
(R_1 = *C*-β-D-glucoside; R_2 = H)

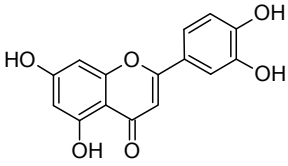


4, eriodictyol-glucoside
(R_1, R_2, R_3, R_4 = OH; *O*- or *C*-β-D-glucosyl in unknown position)

5, eriocitrin
(R_1 = *O*-β-D-glucosyl; R_2, R_3, R_4 = OH)

6, hesperidin
(R_1 = *O*-β-D-glucosyl; R_2, R_4 = OH; R_3 = OCH_3)

8, hesperetin
(R_1, R_2, R_4 = OH; R_3 = OCH_3)



7, luteolin

Fig. 1. Structures of phenolic compounds of *C. intermedia* extract.

A contributing factor to its growing popularity is the body of scientific evidence that honeybush has potential health benefits, including antioxidant, anticancer and phytoestrogenic properties (Joubert et al., 2008a). The greatest demand is for the traditional product, which is 'fermented' (oxidised) to form the characteristic dark-brown colour and sweet flavour. However, this is accompanied by a substantial reduction in the phenolic content of the plant material and extract, as well as a decrease in antioxidant activity (Joubert et al., 2008b), justifying investigating the benefits of unfermented *C. intermedia* for human health.

With T2D being the most common form of diabetes, representing more than 90% of all cases (WHO, 2011), the antidiabetic potential of unfermented *C. intermedia* was investigated using a diet-induced obese insulin resistant (OBIR) rat model. Feeding rats a high fat diet induces a state of insulin resistance associated with impaired insulin-stimulated glycolysis and glycogen synthesis (Kim et al., 2000). The STZ-induced diabetic rat model, following pancreatic β-cell destruction and resulting in insulin deficiency rather than insulin resistance (OBIR model), was used to establish the optimal acute glucose lowering dose of a *C. intermedia* extract. The investigation focused on a hot water extract of *C. intermedia* as it represents normal preparation of the herbal tea, albeit under more severe extraction conditions, but without introducing qualitative changes to composition as is the case for organic solvent extraction.

2. Material and methods

2.1 Chemicals

General analytical grade laboratory reagents were purchased from Sigma-Aldrich (St. Louis, USA) and Merck (Darmstadt, Germany). Authentic reference standards were obtained from Sigma-Aldrich (mangiferin, hesperidin) and Extrasynthese (Genay, France; eriocitrin, luteolin). Isomangiferin was isolated from *C. subternata* (De Beer et al., 2009). Acetonitrile for HPLC analysis was gradient grade for liquid chromatography (Merck, Darmstadt, Germany). HPLC grade water was prepared by purifying laboratory grade water (Continental Water Systems Corp., San Antonio, USA) with a Milli-Q 185 Académic Plus water purification system (Millipore, Bedford, USA).

STZ, fluothane, 50% dextrose solution, metformin hydrochloride and rosiglitazone maleate (Avandia®) were obtained from Sigma-Aldrich, AstraZeneca Pharmaceuticals (Johannesburg, South Africa), Intramed (Johannesburg, South Africa), Rolab (Johannesburg, South Africa) and GlaxoSmithKline (Bryanston, South Africa), respectively.

2.2 Plant material and extract preparation

Cyclopia intermedia shoots (ca 220 kg) were harvested according to normal practice by cutting the shoots sprouting from the rootstock directly above soil level. All the plant material was harvested from a natural stand on Nooitgedacht Farm in the Langkloof area, South Africa. The part of the stem without leaves or with only a few leaves (Fig. 2) was removed before further processing, entailing cutting of the shoots into small pieces (<4 mm) and mechanical drying at 40 °C to less than 10% moisture content (Joubert et al., 2008b), giving ca 50 kg dried plant material. The plant material was pulverised with a Retsch rotary mill before pilot plant extraction. Water heated to >95 °C was added to the pulverised plant material in a 1:10 (m/v)

ratio and continuously stirred for 30 min. (final extract temperature was 70 °C), whereafter the extract was pumped to an in-line continuous centrifuge for removal of the insoluble matter. Following centrifugation, the extract, containing 2.69 g soluble matter/100 mL, was cooled to ca 20 °C using a tubular heat exchanger and aliquots bottled for daily feeding of OBIR rats. The aliquots were stored at -20 °C until use. An aliquot (ca 1000 mL) was freeze-dried in an Edwards Modulyo bench-top freeze-drier (Edwards High Vacuum Ltd, Crowley, UK) for acute dosing of STZ-induced diabetic rats and for phenolic content analysis.

(a) (b)

Fig. 2. (a) *Cyclopia intermedia* plant showing aerial parts (top section of a typical shoot with thin stems and leaves). (b) Harvested shoots before removal of the stem parts without leaves.

2.3 HPLC analysis of extract

HPLC analysis was performed using an Agilent 1200 series HPLC system consisting of a quaternary pump, autosampler, in-line degasser, column oven and diode-array detector (Agilent Technologies Inc., Santa Clara, USA) controlled with Chemstation 3D LC software. Separation was performed on a Zorbax Eclipse XDB-C18 column (150 x 4.6 mm, 5 µm particle size, 80 Å pore size) from Agilent Technologies protected by a guard column with the same stationary phase. The separation was achieved with mobile phases consisting of 0.1% formic acid and acetonitrile using the solvent gradient reported previously (De Beer & Joubert, 2010). The flow-rate and column temperature were maintained at 1 mL/min and 30 °C, respectively. The freeze-dried extract was dissolved in 16% DMSO (ca 5 mg/mL) and filtered using a 33 mm Millex-HV PVDF syringe filter unit with 0.45 µm pore size (Millipore) before injection (20 µL). Compound identification was based on retention times and UV-Vis spectra of authentic standards where available (mangiferin, isomangiferin, eriocitrin, hesperidin, luteolin and hesperetin). An additional peak was identified as an

eriodictyol-glucoside based on similarity of its retention time and UV-Vis spectrum with a compound identified previously using liquid chromatography with mass spectrometric detection (De Beer & Joubert, 2010). An unidentified compound was also observed, which had retention time and UV-Vis characteristics similar to a compound previously detected in several *Cyclopia* spp. (De Beer & Joubert, 2010). Mangiferin, isomangiferin and luteolin were quantified using the peak areas at 320 nm, while the eriodictyol-glucoside, eriocitrin, hesperidin, hesperetin and the unidentified compound were quantified using the peak areas at 288 nm. A calibration series consisting of mangiferin (0.05-2.5 µg injected), isomangiferin (0.05-2.5 µg injected), eriocitrin (0.01-0.8 µg injected), hesperidin (0.01–1.5 µg injected), hesperetin (0.003-0.25 µg injected) and luteolin (0.05-0.35 µg injected) was used for external calibration. The eriodictyol-glucoside was quantified in terms of eriocitrin equivalents, while the unidentified compound was quantified in terms of hesperidin equivalents.

2.4 Animal study

Ethical approval was obtained from the Ethics Committee for Research on Animals (ECRA) of the Medical Research Council of South Africa.

Male Wistar rats, obtained from the Primate Unit of the Medical Research Council (Tygerberg, South Africa), were used throughout the study. The rats were housed individually in wired top and bottom cages, fitted with Perspex™ houses and kept in a controlled environment of 23–24 °C, 50% humidity and a 12 h light/dark cycle.

2.4.1 STZ-induced diabetic rats

Adult male Wistar rats (200-250 g) were injected intramuscularly with freshly prepared STZ [35 mg/kg body weight (BW)] in 0.1 M citrate buffer (pH 4.5) to induce stable non-ketoacidotic diabetic rats. Blood samples were taken from the tail tip 72 hrs after STZ injection and the plasma glucose concentrations determined using a glucometer (Precision Q.I.D, Abbott Laboratories, Johannesburg, South Africa). Rats with fasting blood glucose levels of >15.0 mmol/L were considered diabetic and were selected for the acute dose finding study of the extract.

2.4.2 Acute dosing of diabetic STZ rats with honeybush extract

The efficacy of a single dose of honeybush extract was determined by administering different doses of freeze-dried extract to STZ diabetic rats. Following a 3 hr fast, baseline plasma glucose concentrations of diabetic STZ rats were determined. The freeze-dried honeybush extract (reconstituted in a fixed volume of distilled water yielding e.g. 5 mg/mL for the 5 mg/kg BW dose, etc.) was administered by oral gavage under light anaesthesia (by inhalation of 2% fluothane with 98% oxygen) to four experimental groups of five STZ rats each at doses of 0 (vehicle control), 5, 25 and 50 mg/kg BW. Plasma glucose concentrations were determined hourly over a 6 hr period.

2.5 Chronic treatment of OBIR rats with honeybush extract

The efficacy of honeybush extract to ameliorate diet-induced insulin resistance, characterised by hyperglycaemia, hyperinsulinaemia, hyperglucagonaemia and dyslipidaemia was investigated using OBIR Wistar rats chronically exposed to the extract for 12 wks (described below).

2.5.1 Inducing insulin resistance in Wistar rats

Three-week old weanling Wistar rats (male) were fed a 40% high fat diet (Table 1) and 30% sucrose in their drinking water *ad libitum* for 9 wks. The high fat diet in combination with sucrose induces insulin resistance and obesity with slightly elevated fasting glucose concentrations (Hallfrisch et al., 1981; Krygsman et al., 2010). After 9 wks on the high fat and sucrose diet, blood was collected for baseline glucose and insulin determination. Thereafter the rats were allocated into experimental groups and maintained on the high fat and sucrose diet during the subsequent 12-wk treatment.

2.5.2 Experimental groups

The untreated control consisted of six 12-wk old OBIR rats that were randomly assigned to the control group.

Groups E1 – E5 (honeybush extract treated groups) consisted of five groups of ten OBIR rats each, receiving 538, 1075, 1792, 2150 or 2688 mg/100 mL honeybush extract (hot water soluble solids), respectively, as their drinking fluid, which also contained 30% sucrose (Table 2). The daily fluid intake was measured for each rat and the average amount of liquid

Nutrients	**% Energy**
Protein	15.09
Fat	40.17
Saturated fatty acids	18.27
Monounsaturated fatty acids	11.45
Polyunsaturated fatty acids	5.75
Carbohydrate	44.73
Kcal/g of food	2.06
Kcal/g of sucrose	0.60
Total energy of diet (Kcal/g)	**2.66**

Table 1. High fat diet (HFD) macronutrient and calorific composition.

Group	**Weight**	**Treatment concentration**	**Fluid intake**	**Treatment intake/day**	**Mangiferin intake/day**	**Hesperidin intake/day**
Control	476 ± 20	–	30 ± 2	–	–	–
E1	441 ± 12	538	33 ± 4	77.2 ± 9.6	4.47 ± 0.55	0.27 ± 0.03
E2	456 ± 20	1075	42 ± 5	206.9 ± 27.5	11.99 ± 1.60	0.73 ± 0.10
E3	442 ± 25	1792	31 ± 4	255.1 ± 64.6	14.79 ± 3.74	0.65 ± 0.23
E4	438 ± 21	2150	32 ± 2	299.1 ± 23.5	17.33 ± 1.36	1.06 ± 0.08
E5	457 ± 36	2688	43 ± 2	531.3 ± 61.1	30.79 ± 3.54	1.88 ± 0.22
Met	489 ± 22	–	31 ± 4	22.0	–	–
Rosi	479 ± 26	–	29 ± 2	4.0	–	–

Table 2. OBIR rat body weight (g), extract concentration (mg/100 mL), fluid intake (mL), treatment intake (mg/kg BW), i.e. *C. intermedia* extract (E1-5), metformin (Met) or rosiglitazone (Rosi), as well as equivalent intake of mangiferin (mg/kg BW) and hesperidin (mg/kg BW), for chronic treatment experiment.

consumed per week for each rat was calculated (Table 2). Aliquots of the aqueous honeybush extract were defrosted daily. In the case of E1 to E4 the extract was diluted with water to give the required concentration of hot water soluble solids, while E5 represented the undiluted extract.

The metformin and rosiglitazone treated groups consisted of three OBIR rats each that received metformin hydrochloride or rosiglitazone maleate at dosages of 22 and 4 mg/kg BW, respectively, in distilled water.

2.5.3 Blood parameters

Determination of fasting plasma glucose. The STZ-induced diabetic and OBIR rats were fasted for 3 hrs and overnight, respectively, prior to determining their fasting plasma glucose concentrations. A drop of blood was collected from the tail tip and the plasma glucose concentration determined.

Determination of fasting serum insulin. Rats were anaesthetised by 2% fluothane inhalation with 98% oxygen. Blood was collected from the tail tip into Eppendorf tubes and stored on ice until centrifuged at 2500 rpm for 15 min. at 4 °C. Following centrifugation, the serum samples were stored at -20 °C until analysis. The serum insulin concentration was determined by radioimmunoassay using a rat insulin measurement kit from Linco® Research (St. Charles, USA).

Intravenous glucose tolerance test (IVGTT). Rats fasted overnight were anaesthetised as described above and a drop of blood obtained from the tail tips. This was used for measuring baseline glucose. Glucose (50% dextrose solution), at a dose of 0.5 mg/kg BW, was injected intravenously over 20 sec and glucose measurements taken at 5, 10, 20, 30, 40, 50 and 60 min.

Determination of fasting plasma cholesterol. Rats were anaesthetised as described above and blood, collected from the tail tips, was prepared and stored at -20 °C for analysis. Total cholesterol concentrations were determined by Pathcare Laboratories (Cape Town, South Africa), using a Bayer-Technicon RA 1000 auto-analyser.

2.5.4 Immunocytochemistry and image analysis of the pancreata

*Harvesting of pancreata. A*fter 12 wks of treatment the rats were euthanised by exsanguination under sodium barbital anaesthesia and pancreata harvested. The whole pancreas was removed, fixed overnight in 4% buffered formaldehyde (pH 7.5) and processed into paraffin wax by standard histological methods. Serial 4 µm thick sections were cut for immunocytochemistry.

Immunocytochemistry. Serial wax sections attached onto silane coated slides were de-waxed with xylene and hydrated through descending grades of ethanol into water. Slides were rinsed in 50 mM Tris–buffered-saline (pH 7.4) and double immuno-stained, using anti-insulin and anti-glucagon primary antibodies. Primary antibody binding to insulin or glucagon was detected by avidin D-biotinylated horseradish peroxidase or streptavidin-biotin-complex/alkaline phosphatase conjugated link antibodies. Insulin positive labeling (β-cells) was visualised with fuchsin red and α-cells with diaminobenzidine tetrahydrochloride. Method controls involved omission of the primary antibody (anti-insulin or anti-glucagon).

Image analysis. Both β- and α-cell areas were measured on each section (minimum of ten sections per group). Computer-assisted measurements were taken with a Canon Powershot S40 digital camera (Tochigi, Japan) mounted on an Olympus BX60 light microscope (Tokyo, Japan), attached to a personal computer to capture images. The acquired images were transferred to the computer using remote capture software from Canon. Image analysis was performed with Leica Qwin Plus Software (Cambridge, UK). The ratio of either the β-cell positive or the α-cell positive area to the total pancreas area was calculated. β-Cell and α-cell sizes were calculated by dividing the total area of each of the cell types by the number of nuclei counted.

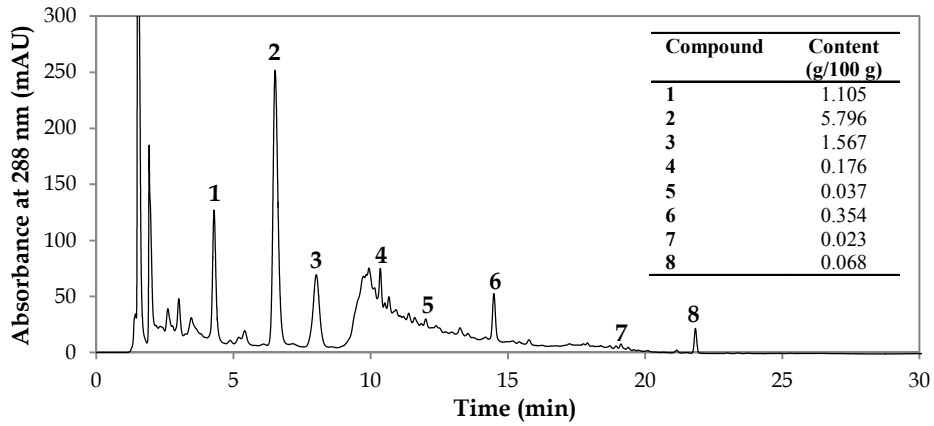
2.6. Statistical analysis

Results were entered into an Excel spreadsheet and statistically analysed. For the acute STZ rat experiment hourly (t = 1–6) glucose concentrations were compared against baseline (t = 0) and the control for each corresponding time point. For the chronic OBIR rat experiments each data point was compared to the corresponding control value using ANOVA with a Dunnet post-hoc test (Prism version 5, Graphpad software®). The values presented are the mean ± SE.

3. Results

3.1 Extract composition

The phenolic compound structures for the major compounds of the *C. intermedia* extract are shown in Fig. 1, the plant shoots in Fig. 2 and the extract HPLC profile and quantitative data in Fig. 3. The major compounds detected included the xanthone isomers, mangiferin and isomangiferin, the flavanone glycoside, hesperidin (hesperetin-7-*O*-rutinoside), and an unidentified compound previously detected in several *Cyclopia* spp. (De Beer & Joubert, 2010). Additionally, a flavone, luteolin, and three flavanones, namely an eriodictyol-



Compound	Content (g/100 g)
1	1.105
2	5.796
3	1.567
4	0.176
5	0.037
6	0.354
7	0.023
8	0.068

Fig. 3. Phenolic profile and phenolic composition (g/100 g) of *C. intermedia* extract (**1**, unidentified compound; **2**, mangiferin; **3**, isomangiferin; **4**, eriodictyol-glucoside; **5**, eriocitrin; **6**, hesperidin; **7**, luteolin; **8**, hesperetin).

glucoside, eriocitrin (eriodictyol-7-*O*-rutinoside) and hesperetin, the aglycone of hesperidin, were also detected in small quantities.

3.2 Animal study

Results of the intake data are summarised in Table 2. The average intake of honeybush extract by the various treatment groups varied from 77 to 531 mg/kg BW. This equals 4.5 to 30.8 mg/kg BW of the major xanthone, mangiferin, and 0.3 to 1.9 mg/kg BW of the major flavanone, hesperidin.

Intramuscular injection of Wistar rats with STZ (35 mg/kg BW) induced diabetes in the rats at an average fasting plasma glucose concentration of 27.8 ± 1.0 mmol/L (data not shown). The acute effects of administering the honeybush extract by oral gavage under light anaesthesia at doses of 0 (vehicle control), 5, 25 and 50 mg/kg BW are shown in Fig 4. The optimal acute oral glucose lowering dose for the honeybush extract in STZ-induced diabetic rats for the dose range tested was 50 mg/kg BW. This was the only acute dose of the extract that significantly reduced the mean blood glucose concentrations relative to the baseline fasting blood glucose concentration. Reductions of 33.5 ± 1.7% ($p<0.05$), 34.3 ± 3.6% ($p<0.05$) and 35.6 ± 3.5% ($p<0.01$) were observed after 4, 5, and 6 hrs, respectively (Fig. 4).

After a chronic 3-month treatment of OBIR rats with aqueous honeybush extract their hyperglycaemic fasting blood glucose concentrations were reduced to normoglycaemic values. In other words, the honeybush extract reduced the fasting blood glucose levels from 12.2 mmol/L of the control to 4.8 – 5.4 mmol/L ($p<0.001$). All extract concentrations were effective. Metformin and rosiglitazone had very similar effects and reduced the fasting glucose to 6.3 mmol/L ($p<0.001$) and 5.6 mmol/L ($p<0.001$), respectively (Fig. 5).

Untreated control OBIR rats had IVGTT peak glucose concentrations of 18.2 ± 1.74 mmol/L. Treatment with the honeybush extract reduced this value to 14.9 ± 0.7 mmol/L (treatment E2, $p<0.05$), 14.8 ± 0.9 mmol/L (treatment E3, $p<0.05$) and 13.5 ± 1.2 mmol/L (treatment E4,

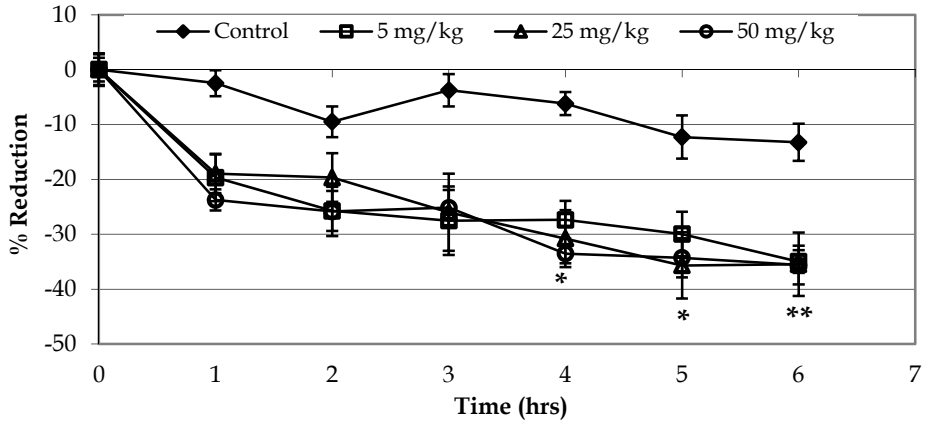


Fig. 4. Acute glucose lowering effect of *C. intermedia* extract in STZ diabetic rats (n=5). Significant differences from baseline indicated with * ($p<0.05$) or ** ($p<0.01$).

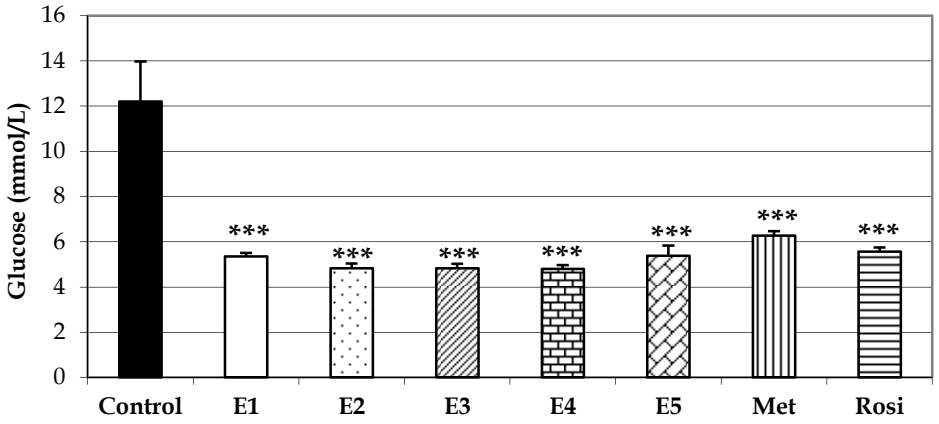


Fig. 5. The effect of chronic treatment with *C. intermedia* extract, metformin and rosiglitazone (see Table 2 for treatment codes and dosage) on fasting plasma glucose concentration of OBIR rats (n=10). Significant differences from control indicated with *** ($p<0.001$).

$p<0.05$), respectively (only E3 is shown for clarity; Fig. 6). The IVGTT area under the curve values were reduced from 670 ± 9 for the control to 474 ± 15 ($p<0.05$) and 496 ± 13 ($p<0.05$) for E2 and E5, respectively (data not shown). E1 (the lowest dose), as well as the known drugs, metformin and rosiglitazone, had no significant effect ($p\geq0.05$) (Fig. 6).

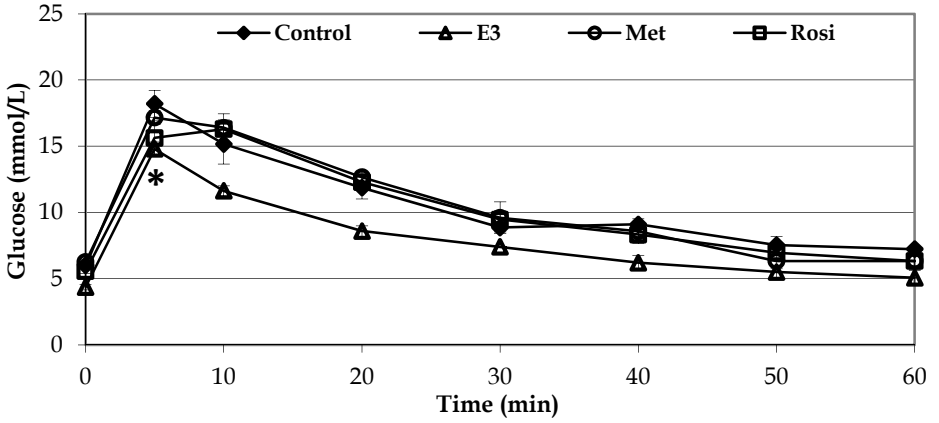


Fig. 6. The effect of *C. intermedia* extract, metformin (Met) and rosiglitazone (Rosi) treatment on intravenous glucose tolerance in OBIR rats (n=10) (see Table 2 for treatment codes and dosage). E3 differs significantly from the control as indicated with * ($p<0.05$).

Treatment of OBIR rats with honeybush extracts, E1 to E5, for 3 months lowered the total plasma cholesterol concentration compared to the untreated rats (2.9 ± 0.3 mmol/L) by 31.6 - 39.1% (Fig. 7). The effect of metformin and rosiglitazone on total cholesterol was not determined.

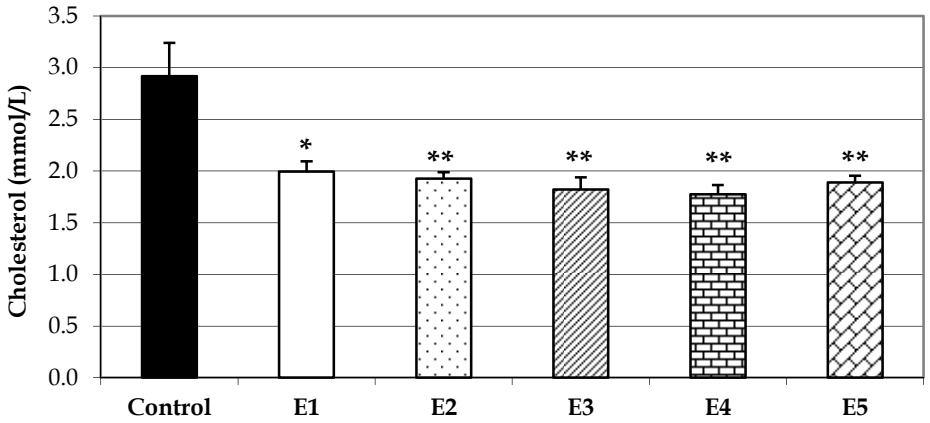


Fig. 7. The effect of chronic treatment with *C. intermedia* extract (see Table 2 for treatment codes and dosage) on fasting total cholesterol concentrations in OBIR rats (n=10). Significant differences from control indicated with * ($p<0.05$) or ** ($p<0.01$).

The average α-cell size in untreated OBIR rats was 106 ± 13.6 μm². Treatment with the honeybush extracts, E1 to E4, reduced the α-cell size to about half, i.e. to 48.4 – 54.9 μm² ($p<0.01$). Metformin and rosiglitazone had similar effects and also reduced the average α-cell size to 41.2 ± 1.9 μm² and 44.0 41.2 ± 1.9 μm², respectively ($p<0.01$) (Fig. 8). No data were available for E5. These changes in the α-cell size were also reflected in the decreased α-cell to β-cell ratio for all treatments (Fig. 9).

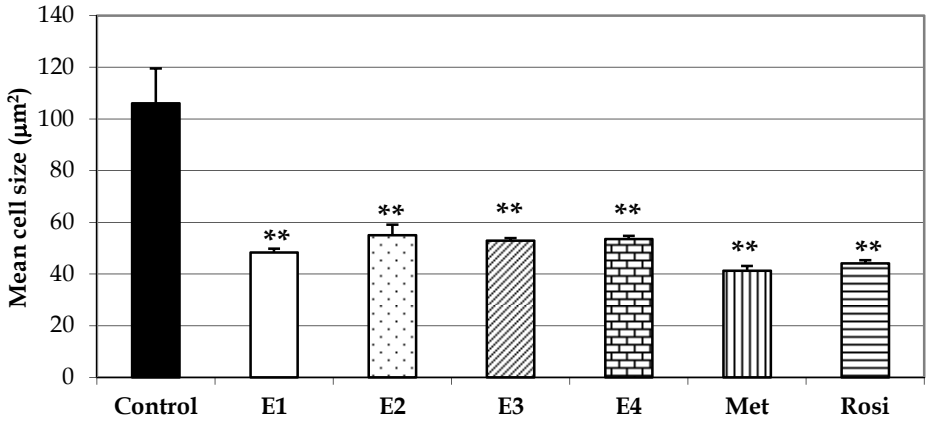


Fig. 8. The effect of chronic treatment with *C. intermedia* extract, metformin and rosiglitazone (see Table 2 for treatment codes and dosage) on mean α-cell size in the pancreata of OBIR rats (n=10). Significant differences from control indicated with ** ($p<0.01$).

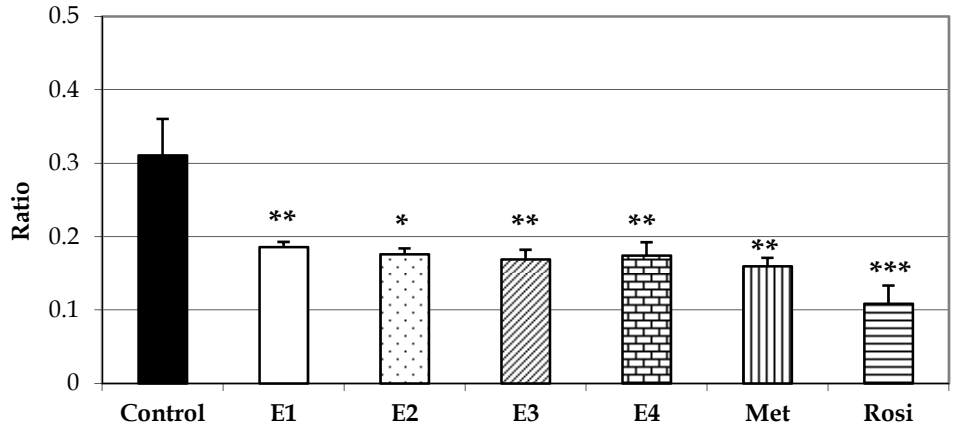


Fig. 9. The effect of chronic treatment with *C. intermedia* extract, metformin and rosiglitazone (see Table 2 for treatment codes and dosage) on α- to β-cell ratio in the pancreata of OBIR rats (n=10). Significant differences from control indicated with * ($p<0.05$), ** ($p<0.01$) or *** ($p<0.001$).

4. Discussion

The content of mangiferin, isomangiferin and the unidentified compound was higher than the average for unfermented *C. intermedia* extracts previously analysed (De Beer & Joubert, 2010; Joubert et al., 2008b), while the hesperidin and eriocitrin contents of the present extract were lower. De Beer & Joubert (2010) detected luteolin only in trace amounts, while no hesperetin was detected. Joubert et al. (2003) found that the plant material (dry mass basis) contain 1.69% mangiferin, while isomangiferin and hesperidin, respectively, comprised 0.22 and 1.76% of the dry plant material. Other phenolic compounds of *C. intermedia* include flavones, isoflavones, other flavanones and coumestans (Ferreira et al., 1998; Kamara et al., 2003). Quantitative differences between the present extract and aqueous hot water extracts analysed previously (De Beer & Joubert, 2010; Joubert et al., 2008b) could be attributed to natural variation and/or selective use of the upper part of the shoot. This has implications for standardisation and efficacy. Testing of more *C. intermedia* extracts, specifically for their efficacy as antidiabetic extracts, is required before more comprehensive claims can be made.

STZ was originally developed as an antibiotic derived from *Streptomyces achromogenes* but it is toxic to pancreatic β-cells. It selectively enters insulin producing β-cells via their GLUT2 glucose transporter proteins, inducing irreparable DNA damage and death of β-cells in a dose dependent manner (Lenzen, 2008). In the Wistar rat, intramuscular injection of STZ (35 mg/kg BW) increased fasting plasma glucose concentrations by ca 500% from the average normoglyceamic concentration of 5.3 mmol/L, resulting in stable non-ketoacidotic T1D diabetic rats. An acute 50 mg/kg BW dose of the aqueous honeybush extract induced a sustained glucose lowering effect from 3 to at least 6 hrs in these STZ-induced diabetic rats. These results are comparable to that of Miura et al. (2001) who assessed the acute antidiabetogenic effect of an extract of *Anemarrhena aspholoides* in a hyperglycaemic KK-Ay

diabetic mouse model. This plant, which contains the xanthones, mangiferin and a 7-glucoside of mangiferin, is used as an Oriential medicine for the treatment of diabetes. After oral treatment the maximal glucose lowering effect of the aqueous *Anemarrhena aspholoides* extract was achieved after 7 hrs. Mangiferin and its glucoside showed similar activity at a dose of 90 mg/kg BW. Mangiferin administered intraperitoneally for 30 days to mildly hyperglycaemic STZ-induced rats at doses of 10 and 20 mg/kg BW ameliorated the diabetic effects including weight loss, hyperglycaemia and hypercholesterolaemia (Dineshkumar et al., 2010). Prior to the latter study, the antidiabetic effects of mangiferin in STZ-induced diabetic Wistar rats were shown at the same doses after chronic treatment for 14 and 28 days (Muruganandan et al., 2005).

In the present study 50 mg/kg BW of honeybush extract, equalling a dose of 2.90 mg mangiferin, was effective at reducing plasma glucose concentrations in our STZ-induced diabetic rat model. Other compounds in the extract could also contribute to the observed hypoglycaemic effect through synergistic or additive effects. Hesperidin, in particular, comprising 0.35% of the honeybush extract, was shown to have a hypoglycaemic effect in marginally hyperglycaemic Wistar rats normalising their blood glucose concentrations after 16 days (Akiyama et al., 2010). Jung et al. (2004), using a spontaneously diabetic C57BL/KsJ-db/db mouse model, showed that a 5-wk supplementation of the diet with 0.02% hesperidin ameliorated the development of hyperglycaemia in these mice. Both Akiyama et al. (2010) and Jung et al. (2004) attributed the hypoglycaemic effect of hesperidin to upregulation of glucose regulating enzymes, in particular glucokinase, which enhances glycolysis and increases glycogen synthesis.

Confirmation of the antidiabetic potential of *C. intermedia* extract in the STZ-induced diabetic rat model was followed up with a chronic study in the high fat diet-induced OBIR rat model. Feeding non-predisposed Wistar rats a high fat (Table 1) and sucrose diet from weanling for three months induces obesity and glucose intolerance. Although these obese rats maintain near normal fasting glucose levels they present with hyperinsulinaemia, hyperglucagonaemia and dyslipidaemia (Buettner et al., 2006; Chalkley et al., 2002; Kamgang et al., 2005). The addition of refined sugars, i.e. sucrose, exacerbates the metabolic aberrations by the disruption of fatty acid metabolism resulting in hepatic and subsequent muscle insulin resistance (Fukuchi et al., 2004). The chronic hyperinsulinaemia associated with insulin resistance increases hepatic lipogenesis due to increased expression of the major lipogenesis gene sterol response element-binding protein 1c (SREBP1c). In these obese rats increasing levels of glucagon fail to regulate the insulin action, leading to increases in hepatic fat accumulation and hypertriglyceridaemia (Buettner et al., 2006). Interestingly, whereas the insulin sensitivity of the SREBP1c pathway is retained, the forkhead box protein O1 (FOXO1) pathway becomes insulin resistant, resulting in decreased hepatic glucose uptake and increased gluconeogenesis. Together the increase in fatty acid synthesis and hepatic glucose release worsens muscle and pancreatic islet insulin resistance (Liu et al., 2011). Ultimately, high fat feeding is directly associated with pancreatic endocrine dysfunction and subsequent morphological changes of the pancreatic islets' endocrine cell ratios and cell sizes. The compensatory response to the increased demand for insulin results in pancreatic β-cell hypertrophy and an increase in α-cell number and volume (Buettner et al. (2007). As the diabetic pathogenesis worsens the β-cell mass declines and the α- to β-cell ratio increases (Liu et al., 2011). Feeding the same 40% high fat diet to pregnant Wistar dams during gestation resulted in offspring with similar increases in α-cell volume, while β-cell

volume decreased (Cerf et al., 2005). In human T2D pancreatic islets, larger islets with an increased proportion of pancreatic α-cells have been shown to have impaired function (Deng et al., 2004). Despite the diet-induced metabolic and morphological aberrations these rats only develop slight hyperglycaemia over time without progressing to overt T2D. The similarities of the diet-induced OBIR rat to the pathophysiology of human obesity and the metabolic syndrome are well established (Buettner et al., 2007) and this animal model was therefore selected to test the possible ameliorating effects of honeybush extract on these metabolic aberrations.

Inclusion of the honeybush extract in an otherwise diabetogenic diet for OBIR rats resulted in normalisation of the pre-existing hyperglycaemia over a wide range of dosages. This confirms that the extract has glucose lowering potential without causing hypoglycaemia. The latter is a potentially undesirable side effect of some T2D agents, specifically the sulfonylureas and meglitinides (Bennett et al., 2011). In addition, the normoglycaemic effect of the honeybush extract was achieved without dietary intervention. The extract proved to be as effective as metformin and rosiglitazone, which are regarded as the gold standards for treating human T2D. The efficacy of the extract at all doses and the lack of a clear dose response could be contributed to the length of the treatment (12 wks) and relative high doses even at the lowest dosage level. The reduction in IVGTT peak values and the area under the curve values in OBIR rats treated with the honeybush extract clearly indicated improved glucose homeostasis. Further, the improvement in glucose control was not associated with significantly increased fasting insulin concentrations. This may suggest that the mechanism whereby the extract elicits its effect on glucose metabolism is independent of insulin, reflecting adaptive mechanisms in the fasted state. This would imply that, due to the low fasting glucose concentrations, β-cells need to release less insulin to maintain glucose homeostasis.

Plasma cholesterol levels of honeybush extract treated OBIR rats were significantly reduced when compared with the plasma cholesterol levels of the untreated control rats. Mangiferin and hesperidin have been shown to lower plasma cholesterol levels of diabetic rats (Akiyama et al., 2010; Dineshkumar et al., 2010; Muruganandan et al., 2005). Mangiferin, apart from lowering total cholesterol, also increases high-density lipoprotein-cholesterol levels and therefore decreases the atherogenic index in diabetic rats (Muruganandan et al., 2005). The hypolipidaemic effect of hesperidin has been attributed to the inhibition of hepatic 3-hydroxy-3-methyl-glutaryl-CoA reductase and acyl CoA:cholesterolacyl transferase, key enzymes in cholesterol synthesis and cholesterol esterification, resulting in the reduction of plasma cholesterol (Bok et al., 1999; Jung et al., 2006). Finally, a potential role of some minor compounds such as the inositol, (+)-pinitol, shown to be present in *C. intermedia* plant material (Ferreira et al., 1998), in the glucose- and cholesterol-lowering effects (Bates et al., 2000; Choi et al., 2009) elicited by the honeybush extract cannot be excluded.

World-wide over 18 million people have died of cardiovascular disease in 2005. WHO has identified high total cholesterol as a major contributing risk factor which is modifiable and thereby could potentially reduce the incidence of cardiovascular disease (Rodgers et al., 2004; Roth et al., 2011). Polyphenols have anti-atherosclerotic properties and are protective against cardiovascular disease. Apart from their plasma cholesterol lowering effects, polyphenols are associated with improved endothelial function and oxidative status

(Badimon et al., 2010). The protective effects of dietary flavonol intake against coronary heart disease mortality in humans was highlighted in a meta-analysis (Huxley & Neil, 2003), indicating a 20% lower risk in individuals with the highest consumption of dietary flavonols. In another study, Peters et al. (2001) suggested drinking three cups of tea (*Camellia sinensis*) a day could reduce the risk of myocardial infarction by 11%. In human trials coronary artery disease patients have shown improved endothelial function and coronary microcirculation following polyphenolic treatment (Hozumi et al., 2006).

The main morphometrical finding following honeybush treatment for 12 wks was a decrease in the α-cell area, and subsequently by inference, total pancreas α-cell volume. The role of glucagon in the pathogenesis of T2D has been largely neglected by researchers. In mice, persistent hyperglucagonaemia induced by glucagon-producing glucagonomas results in T2D (Y. Li et al., 2008). Glucagon secretion by the α-cell is tightly regulated by insulin via insulin receptors at the surface of α-cells. This ensures that normoglycaemia is maintained. However, as insulin resistance increases, as is the case with the OBIR rat model, dysregulation of glucagon secretion by insulin occurs, resulting in persistent hyperglucagonaemia. Morphologically, this results in increased α-cell numbers, α-cell volume and glucagon secretion that worsen the insulin resistance of the liver causing more hyperglycaemia and T2D (Liu et al., 2011). Honeybush extract treatment reduced the average α-cell size and subsequently the total α-cell area leading to an improved α- to β-cell ratio in OBIR rats. The reduction of α-cell size and therefore glucagon secretion can have profound effects on glucagon induced insulin secretion and thereby alleviate the increased stress of hypersecretion on the β-cells (Liu et al., 2011). Similar to our findings, treatment of a high fat diet/STZ T2D mouse model with sitagliptin, a dipeptidyl peptidase-4 inhibitor and a new generation of antidiabetic drug, reversed the increased proportion and distribution of pancreatic α-cells and thereby restored the α- to β-cell ratio following chronic 11 week treatment. As with our study the reduction of the α- to β-cell ratio reflected the overall improvement of the glucose and lipid metabolism (Mu et al., 2006). The fact that metformin and rosiglitazone had a similar beneficial effect on the pancreatic α- to β-cell ratio as the honeybush extract is not surprising. Both metformin and rosiglitazone are established oral drugs for the treatment of T2D. Metformin, an activator of AMP-activated protein kinase (AMPK), not only ameliorates hyperglycaemia but has been shown to have additional beneficial effects on lipid metabolism. Activation of AMPK by metformin increases fatty acid oxidation by inactivation of acetyl-CoA carboxylase and suppression of lipogenesis by inhibiting SREBP-1 expression (Zhou et al., 2001). Rosiglitazone, a peroxisome proliferator activated receptors-γ agonist, has been shown to improve insulin resistance and to have a positive effect on lipid metabolism in pre-diabetic animals by inhibiting malonyl-CoA and thereby increasing fatty acid oxidation in skeletal muscle and liver (Zhao et al., 2009). In addition, rosiglitazone has also been demonstrated to activate AMPK but via a different pathway from metformin (Fryer et al., 2002). Taken together, the positive morphological changes observed support the improved metabolic changes induced by chronic ingestion of aqueous honeybush extract.

5. Conclusion

The efficacy of an aqueous hot water honeybush extract in reducing hyperglycaemia was demonstrated in two different diabetic rodent models (STZ-induced T1D and diet-induced

T2D OBIR rat models). Furthermore, the extract promoted normoglycaemia in the diet-induced T2D OBIR rat model and improved other metabolic aberrations associated with T2D. The high concentration of mangiferin and hesperidin in the extract could be partially responsible for the observed effects.

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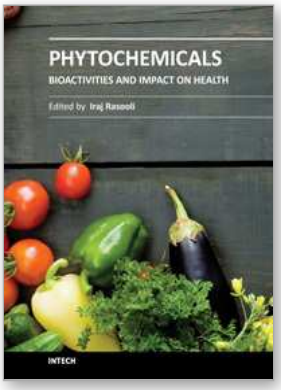

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Among the thousands of naturally occurring constituents so far identified in plants and exhibiting a long history of safe use, there are none that pose - or reasonably might be expected to pose - a significant risk to human health at current low levels of intake when used as flavoring substances. Due to their natural origin, environmental and genetic factors will influence the chemical composition of the plant essential oils. Factors such as species and subspecies, geographical location, harvest time, plant part used and method of isolation all affect chemical composition of the crude material separated from the plant. The screening of plant extracts and natural products for antioxidative and antimicrobial activity has revealed the potential of higher plants as a source of new agents, to serve the processing of natural products.

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